Scientific Note

TLC for the Analysis of Herbal Drugs

A Critical Review of the Status and Proposal for Improvement of Monographs

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ABSTRACT

For the analysis of medicinal plants Thin-Layer Chromatography (TLC) is well suited. Unsurpassed flexibility due to a large number of parameters, which can influence the chromatographic result, is one of the inherent advantages of the method. On the other hand, without standardisation and precise definition of those parameters, results in TLC are difficult to reproduce. As it is currently presented in the European Pharmacopoeia (Ph. Eur.), state of the art features of modern TLC are widely ignored. The following paper is an attempt to point out possible improvements in the general method descriptions as well as in individual monographs. The focus is on optimization and standardisation of experimental details, which can help to increase the reproducibility of the method. Based on theoretical discussions of individual parameters and several practical examples, the advantages of modern HPTLC (high performance TLC) are illustrated and the need for a standardised approach to TLC methodology in new pharmacopoeial monographs is explained.

KEYWORDS

Derivatisation, documentation, high performance thin-layer chromatography, methodology, plate development, sample application, standardisation, thin-layer chromatography.

1. INTRODUCTION

Traditionally TLC has been widely used for the analysis of medicinal plants and it is included as a method for identification in monographs of herbal drugs in most pharmacopoeias throughout the world. The general method descriptions for TLC are usually unspecific and leave much room for individual decisions. Hence the results obtained with such methods can vary considerably and may be difficult to judge. The reproducibility obtained with TLC analyses is often not satisfactory because many parameters that influence the chromatographic result are neglected. There is considerable resistance against any modernization of methodology. Consequently, a general trend is observed to consider TLC as an outdated and old-fashioned technique, which should be replaced by the “more reliable” High Performance Liquid Chromatography (HPLC) or other chromatographic techniques.
Yet TLC offers a number of advantages, which must not be underestimated. Not only for identification of herbal drugs but also for stability test of extracts and finished products as well as during in-process control of the manufacturing process, the possibility of presenting the chromatographic result as an image cannot be substituted [1]. Rapid analysis and low cost per sample are additional benefits.
A principal requirement for increasing the recognition and acceptance of TLC as a competitive, state of the art analytical method is the consequent implementation of a modern standardised methodology, which should be applied to new monographs. This paper presents fundamental aspects of TLC and practical considerations, which are essential to reach that goal. It should be regarded as an invitation for a constructive discussion in the topic.

2. STATE OF TLC IN THE PHARMACOPOEIAS

In the Ph. Eur., TLC is mentioned as a primary tool for identification as part of monographs on all medicinal plants, most extracts and on several synthetic drugs. For the latter, a trend is seen to eliminate TLC in new and revised monographs particularly as a tool for detecting impurities.
Although the general method section describes TLC at a more advanced level than any other pharmacopoeia, the state of the art in methodology and the benefits of today’s technology is still not

reflected. Only the use of pre-coated TLC plates is permitted but no clear distinction is made between TLC and HPTLC layers and no preference is given to either. Guidance concerning several important experimental details (sample application, chromatogram development, derivatisation) is missing and the most important advantage of TLC, the possibility of presenting qualitative chromatographic results in form of an image, is not even mentioned.
Even though it is described as a quantitative method in the general section there are only a couple of monographs featuring quantitative TLC. All assays are to be done by HPLC and even for identification purposes TLC is being replaced successively by other techniques.
The current situation could easily be changed, if a new approach to modern TLC would be taken.

3. PROPOSALS FOR METHODOLOGICAL IMPROVEMENTS

3.1 Plate materials and specifications

At the end of the 1970s, HPTLC adsorbents were introduced on the market. They are characterized by smaller average particle size (5 µm) and a narrow size distribution (2-10 µm). HPTLC layers are more homogenous, have a smoother surface and a higher separation power than conventional TLC plates. Table 1 compares both types.

Table 1: Comparison of plate materials

		TLC	**HPTLC**
Particle size distribution	[µm]	2 – 40	2 – 10
Average particle size	[µm]	10 – 15	5
Layer thickness	[µm]	250	100, 200
Separation distance	[mm]	100 – 150	30 – 70
Optimal separation distance	[mm]	120	60
Running time for optimum separation distance	[min]	30 – 60	7 – 20
Solvent consumption (Twin Through Chamber)	[mL]	25 – 50 (20x20cm)	10 – 20 (20x10 cm) 5 – 10 (10x10 cm)
Detection limit, absorbance	[ng]	100 – 1000	10 – 100
Detection limit, fluorescence	[ng]	1 – 100	0,1 – 10
Comparison of prices per plate (glass back) normalised: 20x20 TLC plate = 1		1 (20x20 cm)	1.2 (20x10 cm) 0.8 (10x10 cm)

It can be assumed that in pharmacopoeial methods prescribing the use of TLC plates, these plates can be substituted by HPTLC material. The resulting chromatogram will typically show improved resolution, less band broadening, and better reproducibility across the plate. The Swiss Expert Committee on Phytochemistry has verified this statement for several methods published in Pharmeuropa for comment. For example, Figure 1 compares the separation of sweet and bitter orange oil on both qualities of stationary phases. It can be concluded, that the use of HPTLC plates is the best and more economical choice.

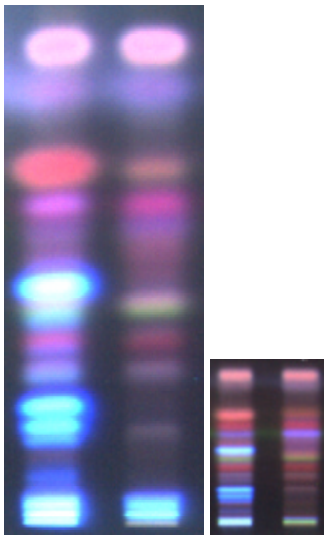


Figure 1 - Separation of sweet (left track) and bitter orange oil (right track) on TLC and HPTLC plates.
Development on the TLC plate (left) over 15 cm requires 45 min, separation over 5 cm on HPTLC material (right) is achieved in 7 min. Mobile phase: ethyl acetate, toluene (15:85 V/V), visualization at 366 nm after derivatisation with anisaldehyde reagent.
Note: All chromatograms have been developed in saturated twin trough chambers.

NOTE: An electronic copy of all figures in colour can be obtained from the authors!

Although it is not permitted to specify brand names in the monographs, it should be noted that plates of different manufacturers can vary significantly with respect to the result obtained with a specific method, yet still pass the system suitability test given in the reagent section of the pharmacopoeia (Figure 2).

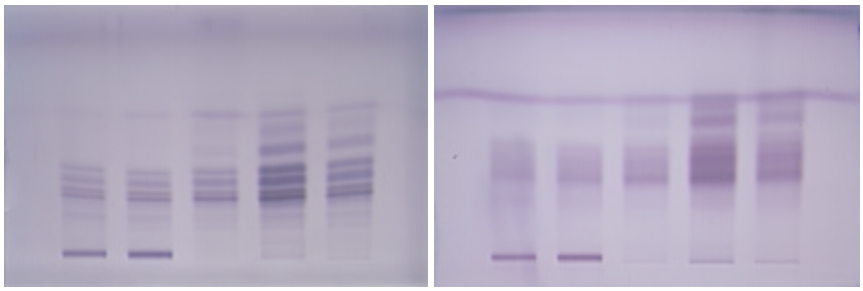


Figure 2 - Separation of several Black cohosh (*Cimicifuga racemosa*) samples on HPTLC Si 60 F254 plates from different manufacturers (left: Merck, right: Macherey&Nagel). Images courtesy of E. Flachsmann AG, Zürich.

Because it is not feasible to design a single test that evaluates the behaviour of the stationary phase for all possible separation problems, the manufacturer of the plate should be specified in the method description. Alternatively, a specific performance test (system suitability test) could be included. As stipulated by the Technical Guide for the Elaboration of Monographs [2], such statement is already required for developmental work submitted to the Pharmeuropa. However, it seems neither practical to include more than one plate material in the method development before finalisation of the monograph, nor does it make sense to include other materials in a test during validation of the method unless such work is all inclusive. An acceptable compromise taking also legal implications into account should be worked out.

3.2 Sample application

All identifications in TLC are primarily based on comparison of migration distances (R_f-values). The quality of the analysis therefore depends on proper positioning of the sample. For quantitative

evaluation, the applied sample volume must also be defined and reproducible. In addition, the quality of the separation depends upon size, shape, and homogeneity of the application zones.
The two principal technical solutions for sample transfer to the plate are contact spotting and spray-on application.
During spot application the solvent of the sample performs “circular chromatography”, which can cause irregular distribution of the sample components across the spot (Figure 3a). After chromatogram development spots may be broad and not symmetric. This can be a particular problem if the sample is dissolved in a solvent of high solvent strength.
Resolution and detection limits of a given TLC system can be significantly improved using the spray-on technique. This way any chromatography during application can be avoided. It is also possible to apply large volumes of samples with low concentration of analyte without loosing quality of separation.
If samples are applied as narrow bands, the visual impression of the chromatogram is improved. If such bands are sprayed, additionally a homogenous distribution of the sample over the entire length of the band is achieved. This is the basis for reliable and reproducible quantitative evaluation. It should be noted, that creating a band by spotting many very small spots side by side or the use of plate with so-called concentration zones will not achieve the same resolution and the zones in the lower R_f-range can be disturbed (Figure 3b-d).

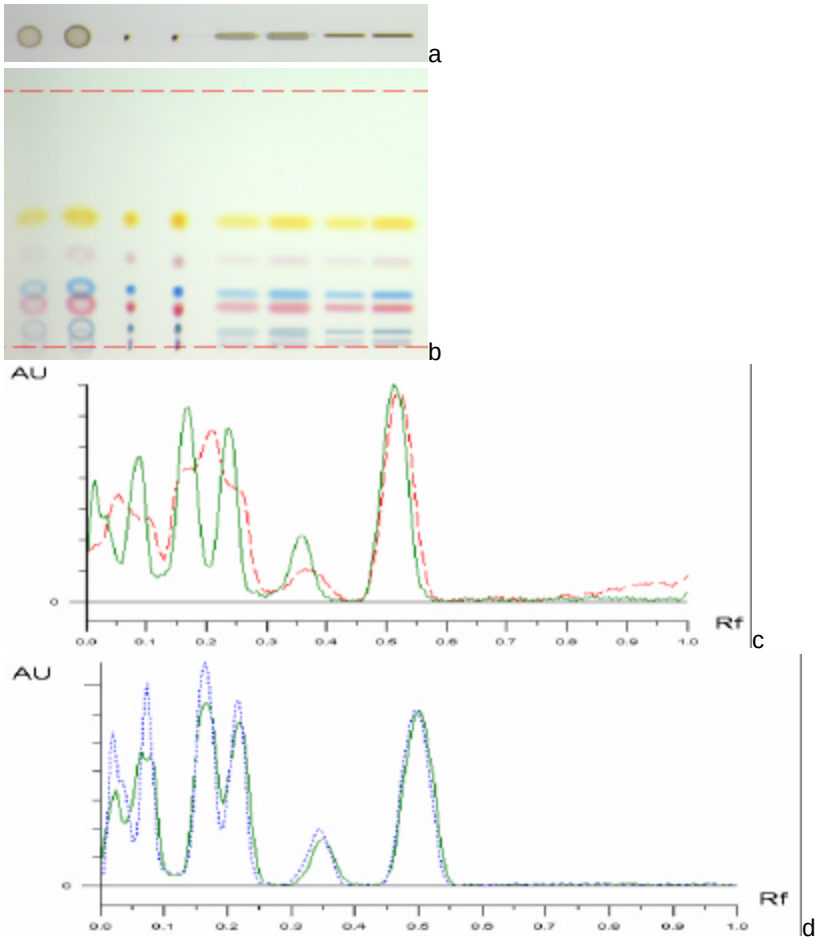


Figure 3 - Influence of different application techniques on the resolution of a test dye mixture in methanol (5 µL and 10 µL each) on HPTLC silica gel 60. Mobile phase: toluene.
Left to right: contact spot, sprayed spot, contact band, and sprayed band;

a) Application position before chromatography;
b) Separation of dye mixture
c) Comparison of contact spot (track 1, dashed line) to sprayed spot (track 3, solid line)
d) Comparison of contact band (track 5, solid line) to sprayed band (track 7, dotted line)

3.3 Development

The outcome of the TLC chromatogram (position, shape and resolution of bands) is dependent on the type and saturation of the developing chamber, as seen in Figure 4. Therefore a method is only reproducible for a specified chamber in a defined configuration and will usually not work in a different system without adaptation. The corresponding theory is described elsewhere [3, 4, 5]. Typically a saturated configuration (tank or horizontal chamber) offers the highest reproducibility, although the chromatogram zones are often more diffuse than in unsaturated or sandwich chambers.

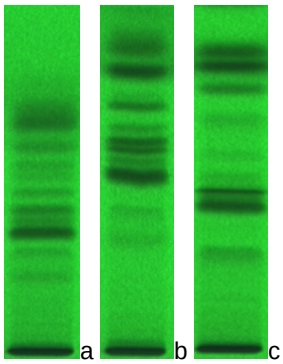


Figure 4 - Chromatogram of *Schisandra* berries developed in a Twin Trough Chamber in a) saturated, b) unsaturated mode, and c) in a Horizontal Developing Chamber in sandwich configuration. Mobile phase: acetic acid, ethyl acetate, toluene (3:33:70 V/V/V), visualization at 254 nm.

In TLC, the velocity of the mobile phase is decreasing during development (Figure 5). Caused by the higher resistance against mobile phase flow of a stationary phase packed densely with fine particles, only short developing distances can be utilized on HPTLC plates.

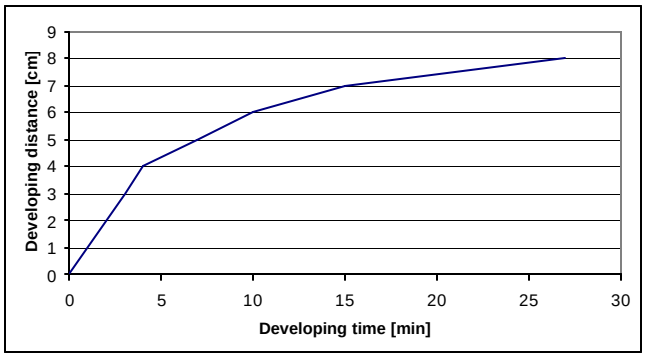
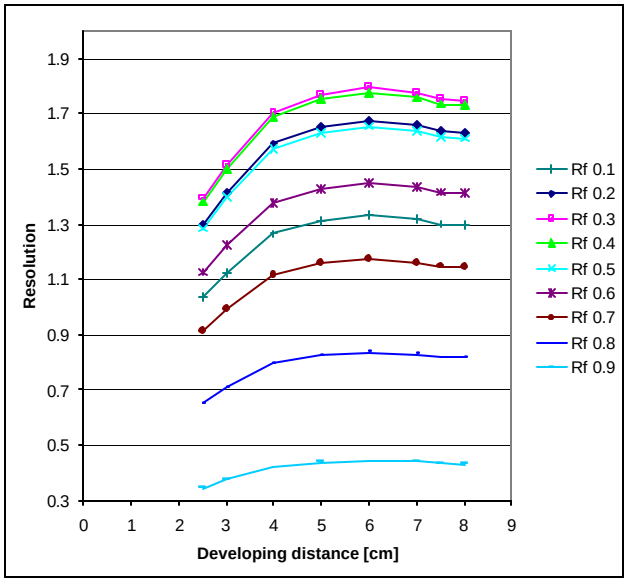


Figure 5 - Relationship of developing distance and the developing time on HPTLC silica gel 60 (experimental values). Mobile phase: ethyl acetate, toluene (5:95 V/V).

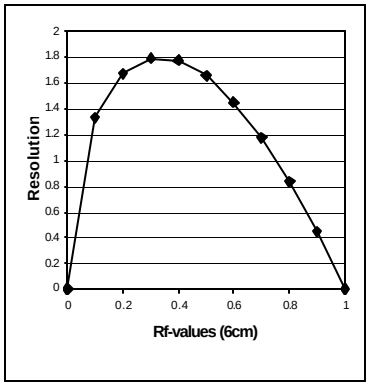
In a given chamber, keeping all other parameters constant, resolution (R_s) of 2 compounds is as well dependent on their relative position in the chromatogram (R_f) as on the migration distance of the front (developing distance).

Figure 6 shows plots of the resolution between 2 components in an HPTLC system with assumed selectivity (α) of 1.5 as a function of the separation distance. Resolution was calculated using the equation (3, p. 666): $R_s = {}^1/_4(\alpha-1)(R_f N)^{1/2}(1-R_f)$.

On HPTLC plates the best resolution is obtained over a developing distance of 5 - 7 cm, with a maximum at 6 cm. For most mobile phases on silica gel the development requires 7 - 20 min. Within a given chromatogram separation is best in the R_f-range of 0.3 - 0.4. Therefore, the solvent strength of the mobile phase should be adjusted so that a critical substance pair is positioned in this range.



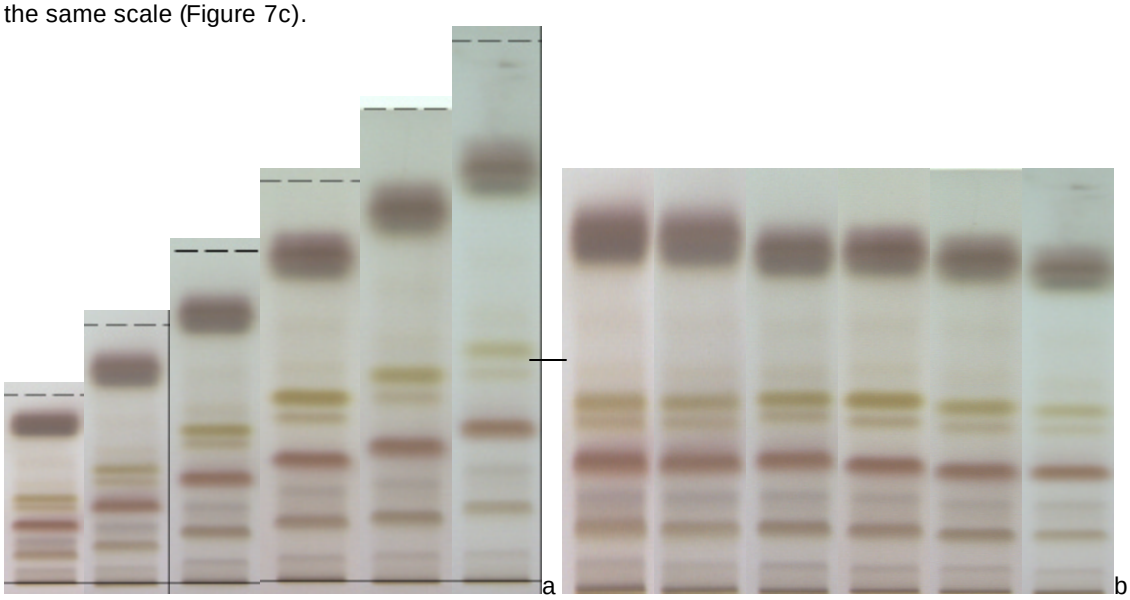
a



b

Figure 6 - Influence of the developing distance (a) and R-values (b) on the resolution: $R_s = {}^1/_4(\alpha-1)(R_f N)^{1/2}(1-R_f)$, selectivity ($\alpha$) 1.5, plate numbers (N) taken from [3, p. 666]

These theoretical predictions can easily be proven experimentally. In Figure 7 the separation of chamomile oil on HPTLC silica gel is presented. Based on the substance pair at R_f 0.4 - 0.5 (arrow) in the chromatograms in Figure 7a, resolution appears to increase as the developing distance is extended. However, if the chromatograms are put on the same scale (Figure 7b), it is seen that the relative position of the 2 components does not change. Resolution still goes through a maximum for 6 cm developing distance. Generally, the R_f-values decrease with extended developing distances. This effect can be explained with the increased loading of the plate with volatile components of the developing solvent. The visual impression can be supported by comparison of the analog curves on the same scale (Figure 7c).



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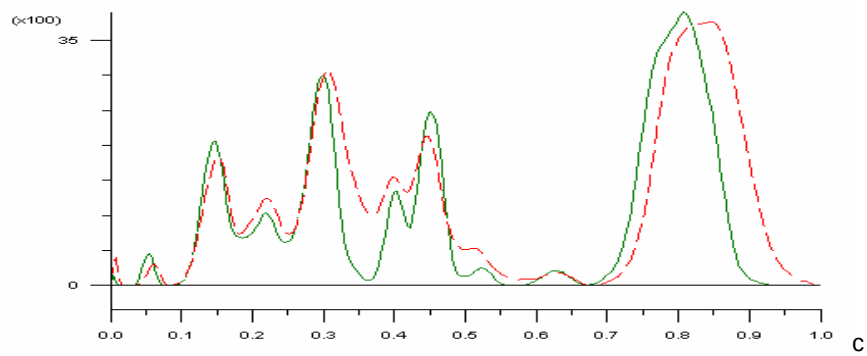

Figure 7 - Separation of chamomile oil on HPTLC silica gel 60. Mobile phase: ethyl acetate, toluene (5:95 V/V), derivatisation by dipping in 10% sulphuric acid in methanol
a) Increasing developing distance (3-8 cm)
b) Chromatograms from a) scaled to the height of track 4 (developing distance of 6 cm)
c) Analog curves of chromatograms over 4 cm (dash) and 6 cm (solid) developing distance

3.4 Derivatisation

Chemical derivatisation to visualize the chromatographic result or to improve detectability can be performed in TLC without difficult operations. Most commonly derivatizing reagents are used in solution. The developed plate is either sprayed with or dipped into such solution. Although it is almost exclusively mentioned in the monographs, spraying is very difficult to standardise unless sophisticated devices are employed. Spraying requires great skills. The amount of reagent, which is transferred onto the plate, as well as the homogeneity of coverage can hardly be described precisely in a method. This makes it almost impossible to reproduce the procedure exactly. Another disadvantage of spraying is the generation of hazardous fumes.

Derivatisation by immersing the plate into the reagent is much easier to perform and to control. The concentration of the reagent as well as the speed and time of immersion can be specified thus ensuring reproducible results. Coverage of the plate is always homogenous enabling reliable quantitative evaluation. No reagent fumes are produced.

Usually chemical derivatisation includes also a heating step, which must be precisely described in the method. For example “Heat at a specified temperature for x minutes or until colors appear” is not a statement sufficient for ensuring reproducible results.

It is obvious, that in the general method section derivatisation cannot be described in detail, however, each monograph should define this step precisely. Parameters, which also have to be specified, include treatment of the developed plate prior to derivatisation, drying conditions of plate after immersion into reagent and any waiting times prior and after a necessary heating step. Figure 8 examines the effects of various experimental details during derivatisation of *Hypericum* extract with Natural Products reagent (NP) / polyethylene glycol (PEG) following chromatography on HPTLC silica gel 60.

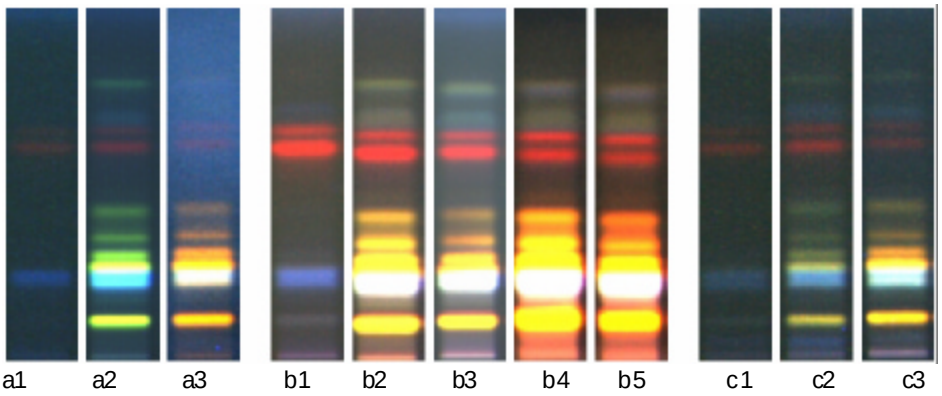

Figure 8 - Effects of experimental details on the result of derivatisation of *Hypericum* extract after separation on HPTLC silica gel 60. Mobile phase: ethyl acetate, dichloromethane, formic acid, acetic acid, water (100:25:10:10:11 v/v/v/v/v), detection at 366 nm.

a: drying developed plate for 3 min with cold air (a1: prior to derivatisation, a2: NP, a3: NP/PEG)
b: drying developed plate for 5 min at 105°C (b1: prior to derivatisation, b2: NP, b3: NP after 30 min, b4: NP/PEG, b5: NP/PEG after 30 min)
c: drying developed plate for 30 min at 105°C (c1: prior to derivatisation, c2: NP, c3: NP/PEG)

3.5 Documentation

A unique feature of TLC, setting the technique apart from all other chromatographic methods is the availability of images to present and communicate the chromatographic result. Today electronic images of TLC chromatograms can be conveniently generated using video or digital cameras or even flat bed scanners.

The verbal description of TLC chromatograms in the current pharmacopoeia is not sufficient to provide a complete, detailed and objective evaluation of the result. For example the result of the identification test for anise oil (covers star anise oil as well) are described in the Ph. Eur. [6]. Under UV 254 nm, the sample shows 2 spots corresponding to anisaldehyde and anethole. After derivatisation with vanillin, under white light, the sample shows spots corresponding to linalool and anethole, there is a violet spot close to the solvent front. In addition *Pimpinella anisum* may show a brown spot above anisaldehyde. Although somewhat improved, because it gives a better impression about relative positions, the presentation of results in form of a table (Table 2) as used by the Pharmeuropa, is still not a suitable alternative to an image of the chromatogram as shown in Figure 9.

Table 2: Description of the chromatographic result in a table for the identification of anise oil according to Pharmeuropa [7]. Additional spots are permitted in the verbal description.

Top of plate	
Anethole: a quenching spot	A quenching spot (anethole)
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Anisaldehyde: a quenching spot	A quenching spot (anisaldehyde)
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Reference solution	Test solution

Top of plate	
	A violet zone (monoterpene hydrocarbons)
Anethole: an orange-pink spot	A an orange-pink spot (anethole)
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Linalool: a blue spot	A blue spot (linalool)
Reference solution	Test solution

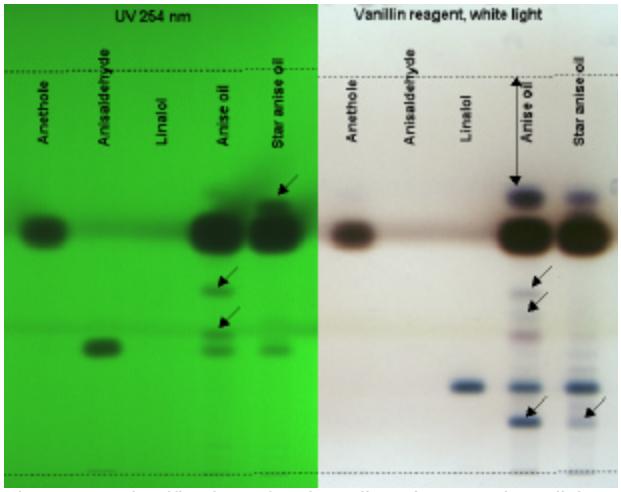

Figure 9 - Identification of anise oil and star anise oil by TLC according to Ph. Eur. [6]. Mobile phase: ethyl acetate, toluene (7:93 V/V)
Left: UV 254 nm, right: after derivatisation with vanillin reagent, white light. All zones marked with arrows are not mentioned in the cited Ph. Eur. monograph. The spot described as "close to the solvent front" appears at R_f 0.75.

It is apparent, that in the given example neither the verbal nor the tabular description fit the result for any of the 2 oils seen on the TLC plate / images. All zones marked with arrows are not mentioned in the monograph [6], nor is the presence of "other" zones permitted. Therefore, the more important question is, whether either of the samples would pass the pharmacopoeial identity test for anise or star anise oil. The images show, that both oils are distinctly different and can easily be discriminated. (Note: Currently the monographs for anise oil and star anise oils are under revision.)

As for all other TLC steps also for documentation using electronic imaging techniques it is of utmost importance that all parameters are precisely specified and validated to ensure reproducible results. In the near future it could be anticipated that electronic images of TLC chromatograms become the basis of an atlas, which would be a useful supplement to the monographs on medicinal plants. It would be a particular advantage of such an atlas, if not only one fingerprint for each plant is provided but also images generated from multiple detections. Furthermore the natural variability of each plant could be illustrated by analysing several representative batches on the same plate. Lastly fingerprints of commonly encountered adulterants could be included as well.

3.6 Standardisation and International Harmonization

In the preceding sections several important parameters have been discussed together with some suggestion for changes to the general method description [8] and individual monographs of the pharmacopoeia. However, it should also be noted, that providing a suitable framework is not enough to utilize the full potential of modern TLC. A new standardised methodological approach must also be taken. Such approach similar to a SOP could be included in the Technical Guide for the Elaboration of Monographs [2]. The example shown in Figure 10 shall illustrate the problem. A method for the identification of *Angelica sinensis* and *Levisticum officinale* by HPTLC was developed by laboratory A, put in writing and transferred to laboratory B for verification. The obtained results are quite similar yet by no means equal, even though both laboratories thought that they followed the method in detail. A closer look reveals that there are some differences in the common practices of the 2 laboratories, which where assumed to be the "right way to do it".

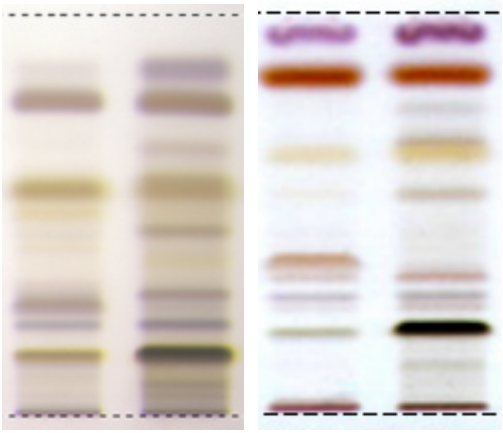

Figure 10 - HPTLC of *Angelica sinensis* (left track) and *Levisticum officinale* (right track). Mobile phase: formic acid, ethyl acetate, toluene (1:10:90 V/V/V)

Experimental details	**Laboratory A (left image)**	**Laboratory B (right image)**
Prew ashing	No	MeOH-$CHCl_3$ (drying in oven 105°, 30 min)
Chamber saturation	20 min (filter paper)	15 min (without paper)
Derivatisation	Dipping	Spraying
Documentation	Video, reflection + transmission	Flat bed scanner
Activity of plate	equilibrium with lab, 40% r.h.	over night (P_2O_5), unknown r.h.

Figure 11 shows the results of the HPTLC identification of Coneflower (*Echinacea* spp). The method closely follows a standardised methodology [9]. It was thoroughly validated according to the guidelines of the AOAC Peer Verified Method Program [10]. The results obtained in two independent laboratories at different times are almost identical [11]. This method was submitted for comments to the Pharmeuropa (Narrow-leaved coneflower root [12], pale coneflower root [13], purple coneflower root [14]).

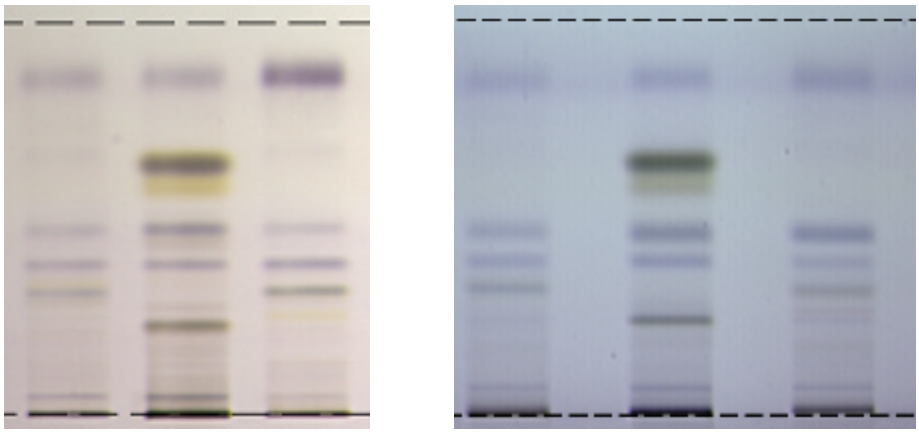

Figure 11 - HPTLC identification of *Echinacea* spp. based on alkylamides. AOAC peer verified method. Mobile phase: formic acid, cyclohexane, ethyl acetate, toluene (0.9:3:6:24 V/V/V/V), derivatisation with anisaldehyde reagent.
Left: laboratory A (plate developed July 2002), right: laboratory B (September 2001). *E. purpurea, E. pallida, E. angustifolia* (left to right tracks)

4. CONCLUSIONS

Based on theoretical and practical considerations it seems necessary to critically review the general method description of the European Pharmacopoeia for Thin-Layer Chromatography. The development of new as well as the revision of existing monographs on medicinal plants should include TLC identification, which is based on a modern standardised methodology. The following key elements are to be considered:

1. TLC methods should generally be based on HPTLC plates.
2. For best qualitative and quantitative results samples should be applied as narrow bands preferably using the spray-on technique.
3. It should be recommended to generally develop HPTLC plates in a saturated chamber over a distance of 6 cm.
4. For methods relying on colour reactions for visualization of the chromatographic result, the derivatisation step must be explicitly described.
5. In addition to a description the result of a method should be provided as a colour image preferably in form of an atlas. Multiple images (such as under UV 254 nm, UV 366 nm, and white light) can significantly increase the certainty of the analytical result. Providing reference material for the products to be tested could also be considered.

Methodological standardisation in TLC is a fundamental requirement for comparability of results.

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